state to a fluorescent state. Only a small fraction of the molecules are activated at any given time; giving a very low probability of nearby molecules being simultaneously activated and forming overlapping blurs in the image. The fluorescent molecules are imaged individually, their positions are determined from the images, and then a new set of molecules is activated to the fluorescent state. However, there is a tradeoff between image quality (improved by minimizing the activation probability per molecule, for fewer overlapping blurs) and speed (improved by increasing the activation probability per molecule, to reduce the risk of image cycles with zero activated molecules). One method of dealing with this tradeoff is to increase the activation probability and use algorithms (called rejection algorithms) that identify and remove spots formed by overlapping blurs from more than 1 molecule.
We performed a theoretical analysis to relate rejection algorithm performance with achievable resolution and image acquisition speed. We predict the existence of a minimum acquisition time independent of algorithm performance, and an algorithm-dependent maximum error rate. We have characterized the performance of commonly-used procedures for identifying multi-molecule spots via their shape (including linear and non-linear curve fitting), and show that procedures of widely varying complexity and speed have comparable performance, pointing to ways of reducing acquisition and post-processing time with optimized rejection algorithms. Additionally, we analyzed errors when molecules produce overlapping blurs and are then bleached. With proper control of activation probability and the photobleaching rate, bleaching can actually be used to enable faster acquisition of an image with subwavelength resolution, with implications for the design of photoswitchable fluorescent proteins.

937-Pos
Imaging Total Internal Reflection Fluorescence Cross-Correlation Spectroscopy (ITIR-FCCS)
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General imaging approaches, in which contrast is not given by time averaged intensities but by other fluorescence parameters, for instance lifetime, anisotropy or parameters of correlation functions, promise to give new insights to biologists. We have recently shown that Fluorescence Correlation Spectroscopy (FCS) can be performed using EMCCD cameras if used with an objective type total internal reflection illumination scheme. This so called Imaging Total Internal Reflection-Fluorescence Correlation Spectroscopy (ITIR-FCS), allows the measurement of autocorrelation functions (ACF) on thousands of pixels independently on 2D surfaces. In this work we extend this technique to ITIR-FCCS (Fluorescence Cross-correlation Spectroscopy) to perform spatial cross-correlation for the measurement of general translational processes. A generalized expression was derived for auto- and cross-correlations of arbitrarily shaped areas on a CCD for diffusion and flow processes. ITIR-FCCS was able to precisely and accurately determine flow velocities and diffusion coefficients of model systems.
To address the translational processes and the organization of cell membranes we calculated the differences between spatial forward and backward cross-correlations to yield so called ΔCCF images. The ΔCCF imaging approach was successfully demonstrated on GUVs and mixed lipid bilayers by demarcating phase boundaries. It was then used to track the changes in heterogeneity of two cell membrane markers, a liquid-ordered phase marker (sphingolipid binding domain (SBD) derived from the amyloid peptide Aβ) and a liquid-disordered phase marker (DiI) on live neuroblastoma cells under conditions of cholesterol depletion and cytoskeletal disruption. Our findings from auto- and cross-correlation and ΔCCF analysis indicate that SBD is influenced by the cholesterol content and to a larger extent by the integrity of the cytoskeleton. DiI, by contrast, shows little dependence on both.

938-Pos
Optical Nanoscopy Far-Field Approaches to Cellular and Molecular Biophysics
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Fluorescence optical far-field microscopy fostered the design and realization of crucial experiments in cellular and molecular biophysics, although the limited spatial resolution dictated by diffraction. Recently, an emerging family of fluorescence microscopy approaches exploiting the photo physical properties and the switching abilities of fluorescent markers allowed to achieve the surpassing of the diffraction barrier down to 10 nm resolution scale. Super-resolution microscopy and optical nanoscopy are the modern terms related to optical far-field methods opening a new window for the understanding of molecular interactions within the biological cell (A. Diaspro (ed.) (2009) ''Nanoscopy and Multidimensional Optical Fluorescence Microscopy '', Chapman and Hall). Within this framework, focusing on the saturated depletion of the markers' fluorescent state by stimulated emission we have pointed our attention to different modalities for realizing STED (stimulated emission depletion) approach. In particular we are interested in the excitation modalities (including phase modeling, intensity control and scanning speed) and in the possible photo-bleaching/toxical effects as function of the light intensity levels needed. To this end we are working on the ''classical'' solution using ps laser pulses both using white light laser generation and multi-photon based schemes as compared to the continuous wave (CW) excitation and depletion achieved by means of CW laser sources. In parallel we are also approaching optical super-resolution using the FPALM (fluorescence photoactivatable localization microscopy) scheme coupled to two different ways for switching on the fluorescent proteins involved. The former being classical, in order to have a comparison with the STED approach in terms of possible photo-bleaching and photo-toxical effects, and the latter based on the utilization of the single plane illumination microscopy (SPIM) concept to extend far-field optical nanoscopy methods to large samples.

939-Pos
Recognition of Protein Binding Events by Polarity-Sensitive Probes
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Polarity-dependent fluorescent probes are recently attracting interest for high-resolution cell imaging. The fluorescence enhancement of the solvatochromic dye, ideally located in a domain where polarity changes occur upon binding, allows for a fine detection of molecular recognition events even between non overexpressed proteins.1 We developed a toolbox of new solvatochromic coumarin derivatives, characterized by a donor-(coumarin core)-acceptor structure, tailored to in vivo imaging applications.
After a preliminary screening by computational methods, we adopted a synthetic procedure tuneable on the substitution patterns to achieve. Our probes possess excellent fluorescence quantum yields (up to 0.95), high molar extinction coefficients (up to 46,000 M-1cm-1), and large Stokes shifts. Furthermore, they display strong solvatochromism, being almost non emissive in water and very fluorescent in less polar media (up to 780-fold enhancement in brightness). When tested on cultured cells, the developed coumarins resulted not harmful and their photophysical properties were unchanged compared to free solution. Due to both their strong solvatochromic properties, and their lipophilic character, the coumarin did fluoresce only in the most lipophilic environments of the cell. In particular, colocalization experiments with standard markers evidenced staining in ER, membranes and lysosomes, depending on the chemical structure of the solvatochromic probe.
Finally, one compound (3-benzothiazenyl-4-ciano-6,7-dimethoxy coumarin) showed monoexponential decay of fluorescence with a lifetime which is linearly dependent on solvent polarity. This feature promotes its use as ratiometric indicator of cell polarity at nanoscale level. The prepared compounds are remarkable tools to investigate subtle biochemical processes in the cell environment after appropriate conjugation to biomolecules, and at the same time constitute the basis for further engineering of a new generation of biosensors.
1) Nalbant, P.; Hodgson, L.; Kraynov, V.; Toutchkine, A.; Hahn, K. M. Science 2004, 305, 1615-1619.

940-Pos
Superresolution Microscopy with Conventional Organic Fluorophores
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Fluorescence microscopy is a sensitive and non-invasive tool to study biomolecular structure and interactions. However, a diffraction limit resulting from the wave nature of light limits resolution to ~200 nm in lateral and ~700 nm in axial direction.
Out of a large set of methods that bypass the resolution limit and open the door for diffraction resolution microscopy, many of them rely on the use of photoactivatable or photoswitchable molecules, combined with precise single-molecule localization and image reconstruction. This concept has recently been extended to a large set of commercially available fluorophores (1-3). Key issues that need to be addressed are (i) controllable switching rates, (ii) live-cell

compatibility, (iii) one-wavelength switching, (iv) labeling densities and (iv) video-rate imaging with subdiffraction resolution.
(1) Heilemann et al., Angew. Chemie, 47, 6172-6176 (2008)
(2) Heilemann et al., Angew. Chemie, 48, 6903-6908 (2009)
(3) Heilemann et al., Laser & Photonics Review, 3, 180-202 (2009)

941-Pos
Optimizing Super Resolution Microscopy
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Super-resolution microscopy is often done by imaging isolated fluorescent probes as diffraction-limited spots with objective-type total internal fluorescence (TIRF) microscopy. The centers of these spots are commonly, sub-optimally located by least-squares fitting a 2D Gaussian to each spot's intensity distribution. Here we give the optimal localization procedure based on the true point spread function (PSF) known from wave optics. From a single focused image of a fluorophore molecule with fixed or time-resolved spatial orientation, we estimate the fluorophore's position and orientation using maximum likelihood estimation. We achieve the highest possible precision, given by Fisher's information limit. In the same manner, optimal localization is demonstrated for isotropic distributions of dipoles, e.g. fluorescent beads, excited by the evanescent wave produced in TIRF. Using this as a baseline, we compare a number of estimators and demonstrate that (i) for a 2D Gaussian, the unweighted least-squares fitting squanders one third of the available information, and weighted least-squares fitting is unreliable; (ii) a popular formula for the localization error of a 2D Gaussian fit exaggerates its precision beyond Fisher's information limit; (iii) maximum likelihood fitting of a 2D Gaussian is, on the other hand, practically optimal. We also present new, reliable formulae for the precisions of the various localization methods.

942-Pos
Fluctuation Analysis with the Spinning Disk Confocal Microscope
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Analysis of the fluctuations in time and space of confocal images has the potential to provide information about molecular diffusion and molecular interactions directly in live cells. Fluctuation image analysis has been commonly done in the laser scanning microscope. In the slow regime, when the fluctuations are slower than the frame rate, the time correlation between the same pixel in different frames of an image stack provides all the information about diffusion and brightness. In the fast regime, for example for molecules diffusing in the cytoplasm, the frame rate is too slow to follow the fluctuations due to diffusion. In the raster scan confocal microscope, these fluctuations are detectable because of the correlation of the intensity with the next pixel in the same line or in the next line. In fluctuation spectroscopy an important parameters is the sampling time that must be shorter that the time of the decay of the fluctuation. In the spinning disk confocal microscope, the sampling time at each pixel is very short. However, in the normal data acquisition protocol of the spinning disk microscope the intensity at one pixel is averaged with the intensity at the same pixel after the disk has performed several rotations. In this work we triggered the camera acquisition so that each pixel is visited only once per frame acquired. While we are observing fluctuations due to fast moving bright particles, the fluctuations due to dim particles seem to be buried in the noise of the system. We are investigating the origin of this extra noise and developing methods to characterize it so that it can be properly subtracted.
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943-Pos
A Simple System for Long-Term 3D Tracking of Quantum Dot Probes in Live Cells
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The intracellular signaling function of G-protein coupled receptors and tyrosine kinase receptors is governed in part by their transport dynamics on the cell surface and within the cell cytosol. Fluorescent nanocrystal quantum dot (QD) probes are especially well suited for investigation of the spatial dynamics of these membrane receptor proteins, including their membrane diffusion, internalization, and intracellular transport. The bright and relatively photostable fluorescence of QDs have enabled dynamic tracking of single or discrete groups of receptors in live cells, but a major limitation in obtaining long-term information on receptor dynamics is that QD probes can only be observed for as long as they remain within the depth of field of the microscope. Because receptors often diffuse or are actively transported out of the focal imaging plane, measurements of QD trajectories are limited in time and z location.
We have overcome this limitation by implementing a 3D tracking system to track receptors for extended durations. Our 3D tracking system is simple and flexible, requiring only an epifluorescence microscope, computer control of a piezo-driven stage and an EMCCD camera. We demonstrate 100-200 nm z-position accuracy over a 10 micron depth for 10s of minutes, with temporal resolution of 7.2s per (x,y,z) coordinate. These capabilities allow measurement of QD probe positions over whole cells for durations relevant to the long-term signaling dynamics of membrane protein receptors. We will present the application of this system to measuring the spatial dynamics of QD-membrane receptor probes for long durations in live cells.

944-Pos
High-Pressure Microscopy for Modulating the Structure and Function of Biomolecules
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Protein hydration is an important factor for structural formation and enzyme activity. To study the effects of hydration, it is desirable to monitor protein structures or biological activities by modulating the intermolecular interaction between protein and water molecules. Application of pressure is one of the powerful methods for enabling the modulation of protein hydration. Here, we report a novel microscopy for visualizing the pressure-induced changes in the structure and function of biomolecules. We have developed a high-pressure chamber (Nickel alloy, hasteloy C276, 60 à- 50 à- 20 mm), which was available up to 2,000 bar. The chamber was mounted on a commercially available microscope equipped with a long working distance objective lens. The microscope apparatus enabled to observe bright-field, phase-contrast, dark-field and epi-fluorescent images at high-pressure conditions. We studied the effects of pressure on the structure and function of cytoskeletal proteins using the kinesin-microtubule complex as a model system [1]. Under high-pressure conditions, taxol-stabilized microtubules were shortened from both ends at the same speed. The sliding velocity of kinesin motors was reversibly changed by pressure and reached the half-maximal value at ~1000 bar. Further analysis showed that the pressure mainly affects the stepping motion, but not the ATP binding reaction. The application of pressure is thought to enhance the structural fluctuation and/or association of water molecules with the exposed regions of the kinesin head and microtubule. These pressure-induced effects could prevent the kinesin motors from completing the stepping motion.
[1] Nishiyama *et al.*, *Biophys J.* **96**(3) 1142-1150 (2009).

945-Pos
Particle Image Cross Correlation Spectroscopy (PICCS)
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In vivo studies of the dynamics of single molecules and particles have produced a wealth of biological insights. The diffusion behavior of a membrane receptor, for example, reveals the structure of the plasma membrane. In recent years we have developed an analysis technique to quantify single molecule translational movement on a nm length and ms time scale (Particle Image Correlation Spectroscopy (PICS), Semrau, Schmidt., Biophys. J., 2007).
The insight gained from experiments on a single molecular species is however limited. No biomolecule operates on its own and often it is the very interaction between different types of molecules which is biologically most relevant. To that end we further developed PICS for experiments with two differently labeled molecular species. Particle Image Cross-Correlation Spectroscopy (PICCS) allows us to unambiguously measure molecule colocalization, even at large molecule densities and down to a length scale of 10 nm. To demonstrate the method's power we studied the intracellular transport of the morphogen Dpp enclosed in endosomes. Dpp establishes a gradient in the wing imaginal disk of fruit fly larvae, providing positional information to cells. Using PICCS we found that 52% of apical Dpp is in early endosomes and that early endosomes contain 1.9 times as much Dpp as other endosomes. Our data suggests that Dpp resides shorter in early endosomes compared to late/recycling endosomes.
PICCS makes it also possible to push the limits of the time scales on which molecular movement can be measured. By labeling one molecule with two spectrally resolvable fluorophores we can follow the dynamics of the molecule on a 100 μs time scale.
To summarize, PICCS opens up a whole new range of *in vivo* single molecule experiments: Molecular correlations and dynamics can be measured with unprecedented accuracy and temporal resolution.